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Non-invasive strategies for stimulating endogenous repair and regenerative mechanisms in the damaged heart

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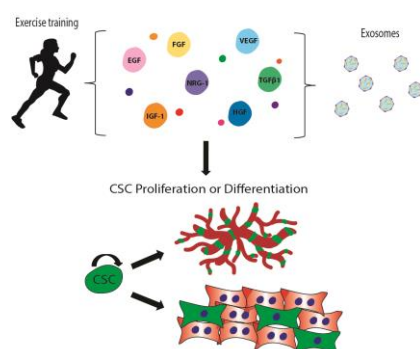
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Graphical abstract



Abstract

The adult myocardium, including human, harbours a population of resident multi-potent cardiac stem cells (CSCs), which when stimulated under the right conditions can give rise to new cardiomyocytes and vasculature. Elucidation of the cellular and molecular mechanisms that govern CSC biology and their role in myocardial regeneration will allow the design and development of optimal therapeutic interventions. It is now evident that different growth factors and cytokines govern CSC survival, proliferation, migration and differentiation, as well as playing a role in activating cardiac repair mechanisms such as improving angiogenesis, cardiomyocyte survival and limiting fibrosis. This review article will summarize the evidence for a role of VEGF, NRG-1, IGF-1, HGF, EGF, FGF and TGF- β 1 in modulating the repair and regeneration of cardiac tissue. It will also discuss the use of exosomes and exercise training as interventions to stimulate the endogenous repair and regenerative mechanisms in the damaged heart.

Abbreviations

BMP - Bone morphogenetic protein

cMRI - Cardiac magnetic resonance imaging

CSC - Cardiac stem/progenitor cell

CABG - Coronary artery bypass graft

CAD - Coronary artery disease

Cnx43 - Connexin-43

CTnI - Cardiac troponin I

DKK 1 - Dickkopf-related protein 1

EGF - Epidermal Growth Factor

EGFR - Epidermal growth factor receptor

EV - extracellular vesicle

FGF - Fibroblast growth factor

GMP - Good manufacturing practice

HF - Heart failure

HGF - Hepatocyte Growth Factor

IGF-1 - Insulin Growth Factor 1

LVEF - Left ventricular ejection fraction

miRNAs - MicroRNAs

MSC - Mesenchymal stem cell

MVB - Multivesicular body

MI - Myocardial infarction

NRG-1 - Neuregulin-1

qRT-PCR - Quantitative reverse transcription polymerase chain reaction

ROS - Reactive oxygen species

SMC - Smooth muscle cell

SP - Side population

TGF- β 1- Transforming Growth factor- β 1

VEGF - Vascular endothelial growth factor

VSMC - Vascular smooth muscle cell

Keywords

cardiac stem/progenitor cells, cardiac repair and regeneration, VEGF, IGF-1, NRG-1, HGF, EGF, FGF, TGF- β 1, proliferation, migration, survival, differentiation, exosomes, exercise training.

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1. Growth factors and cytokines that promote cardiac repair and regeneration

Cardiac regeneration therapies need to encompass neomyogenesis, neoangiogenesis, scaffold/extracellular matrix integrity, and CSC survival, self-renewal and differentiation.

In the long-term the field aims to move towards cell-free, protein-based therapies. Therefore, various growth factors and cytokines have been singled out as candidates for therapeutic cardiac regeneration. As the list of growth factors and cytokines expands so too does our knowledge of growth factor-mediated regenerative potential.

1.1 Vascular endothelial growth factor (VEGF)

VEGF is the cornerstone cytokine involved in promoting neo-vascularisation thus has been the focus for the treatment of heart disease [1, 2]. Initial observations in animal models suggested that recombinant VEGF protein administration could confer beneficial effects [3]. However, promising results from pre-clinical studies were not effectively translated in human clinical trials, where intracoronary protein administration in patients was found to be ineffective [4]. This is now known to be due to the short half-life of VEGF *in vivo* coupled with insufficient myocardial uptake after coronary infusion. It was thought that these issues could largely be overcome through delivery of the VEGF gene rather than protein therefore subsequent studies focused on gene therapy approaches, with pre-clinical efforts again demonstrating improved cardiac function following gene delivery [5, 6]. Pilot gene therapy studies targeted patients with coronary artery disease and demonstrated a favourable safety profile along with an improvement in cardiac function [7] however more-rigorously designed clinical trials, such as the EUROINJECT ONE [8] and NORTHERN [9], did not report any significant clinical benefit. Co-administration of VEGF with stem cells or genetic manipulation to increase the VEGF secretory capacity of stem cells were also investigated. One study delivered VEGF-A¹⁶⁵-transfected mesenchymal stem cells (MSCs) into damaged rat hearts and showed a reduced infarct size, increased ejection fraction, and capillary density [10], whilst the MyStromalCell trial utilised VEGF-A¹⁶⁵-stimulated adipose-derived stromal stem cell transplantation in patients with chronic myocardial ischemia [11]. Delivery of VEGF-A in combination with various scaffolds have also achieved much greater success in stimulating angiogenesis and restoring cardiac function [12, 13]. Together this would strongly suggest that controlled and targeted delivery of VEGF needs to be further developed to achieve efficacious clinical outcomes in terms of improving neovascularisation.

1.2 Neuregulin 1 (NRG-1)

NRG-1 has been implicated in stimulating cardiac repair and regeneration [14, 15]. It is thought that NRG-1 imparts functional benefits by activating and increasing c-kit^{POS} endogenous cardiac stem cell (CSC) proliferation [15], inducing cardiomyocyte replacement [16], protecting cardiomyocytes from apoptosis and improving mitochondrial function [17]. More recently, D'Uva *et al.* demonstrated that transient induction of the NRG-1 receptor, ErbB2 following myocardial infarction (MI) in adult mice led to improved heart function, reduced scarring and increased cardiomyocyte proliferation, compared with mice that did not receive activated ErbB2 [18]. Two Phase II human trials have reported that daily infusions of recombinant human NRG-1 (rhNRG-1) demonstrated improved and sustained hemodynamic measurements in patients with chronic heart failure [19, 20].

1.3 Insulin Growth Factor 1 (IGF-1) and Hepatocyte Growth Factor (HGF)

IGF-1 modulates multiple signaling cascades, resulting in a potent proliferative signal that blocks apoptosis and stimulates growth in multiple different cells and organs [21]. Interestingly, experimental evidence shows that reduced IGF-1 signalling in animals is associated with lifespan extension [22].

Skeletal muscle-restricted expression of IGF-1 increased bone marrow and muscle satellite stem cell pools, providing evidence for its effects on cell proliferation and subsequent increased muscle mass in vivo [23]. Comparable findings have been reported in the heart, where cardiac overexpression of IGF-1 increased the abundance of c-kit^{pos}/Sca-1^{pos} side population cells (SP) in the bone marrow and CD34^{pos} SP cells in the heart [24]. Moreover, IGF-1 mediated the release of cytokines involved in activating the SP cells therefore promoting cross-talk between the heart and bone marrow leading to increased capillarisation following injury [25]. Furthermore, Santini et al. showed in post-infarcted mice, which express the transgene of the locally acting IGF-1 in the myocardium (mIGF-1), improved myocardial function, which was coupled with a modulated inflammatory response, increased anti-apoptotic signalling and an increased number of newly-formed cardiomyocytes. The origin of these newly-formed myocytes was not elucidated, but it is likely they are the product of endogenous CSC differentiation. We have documented that when c-kit^{pos} GATA-4^{high} CSCs are co-cultured with rat cardiomyocytes, cardiomyocyte survival and contractility is enhanced through a paracrine mechanism of increased IGF-1 signalling [26]. Over-expression of myocardial IGF-1 leads to prevention of cardiomyocyte attrition during ageing with a concomitant increased survival and number of CSCs. This resulted in increased myocyte formation governed by the PI3K-Akt signalling pathway [27]. It is proposed that the beneficial paracrine effect of IGF-1 in the heart acts via JNK1/SirT1 signalling [28], through interference with reactive oxygen species (ROS) generation and oxidative DNA damage reversal by homologous recombination [29, 30]. Elucidating the mechanistic role for IGF-1 in myocardial regeneration will enable the development of strategies to stimulate endogenous repair and regeneration in a clinical setting.

HGF and its associated receptor c-met were originally identified as mediators of liver regeneration [31]. However, HGF has now been linked to various tissues and has reported mitogenic and anti-apoptotic capabilities [32]. The role of endogenous HGF in cardioprotection has been documented by Nakamura et al. who identified the expression of both HGF and c-met in cardiomyocytes and demonstrated that plasma levels of HGF rapidly increase in response to ischaemia/reperfusion injury. Neutralization of HGF in vivo was shown to result in cardiac dysfunction, while administration of exogenous HGF reduced apoptosis, decreased infarct size and improved cardiac function, compared with controls [33, 34].

In terms of myocardial regeneration, HGF stimulates migration, proliferation and differentiation of CSCs [35, 36]. We have shown that intracoronary administration of IGF-1 and HGF, in doses ranging from 0.5 to 2µg HGF and 2 to 8µg IGF-1, 30 minutes after MI during coronary reperfusion in the pig triggers a regenerative response from the c-kit^{pos} endogenous CSCs. This effect is potent and produces physiologically significant regeneration of the damaged myocardium without the need for cell transplantation [35]. IGF-1/HGF, in a dose-dependent manner, improved cardiomyocyte survival, reduced fibrosis and cardiomyocyte reactive hypertrophy, increased CSC migration, proliferation

and cardiomyogenic and microvasculature differentiation. These cellular changes were correlated with a reduced infarct size and an improved ventricular segmental contractility and ejection fraction at the end of the follow-up assessed by cardiac magnetic resonance imaging (cMRI) [35].

HGF mediated myocardial regeneration has also been documented through transplantation of human adipose tissue-derived stem cells overexpressing HGF in a rat model of MI. Twenty eight days after MI, results showed an increased left ventricular ejection fraction (LVEF) along with attenuated fibrosis and improved angiogenesis [37]. More recent advances in gene therapy have led to the safety and feasibility of delivering HGF (via adenoviral vectors) to patients with severe coronary artery disease in first-in-man clinical trials [38].

1.4 Epidermal Growth Factor (EGF)

In cardiovascular biology EGF has been implicated in many biological processes including regulation of blood pressure, angiogenesis, neointimal hyperplasia, atherogenesis and cardiac remodelling [39]. In the heart, the main source and target of EGF ligands are endothelial and smooth muscle cells (SMCs) [40]. Transgenic mice expressing dominant negative epidermal growth factor receptor (EGFR) in cardiomyocytes suffered from cardiac hypertrophy and cardiac dysfunction [41], and mice that had a smooth muscle targeted deletion of EGFR, had reduced diastolic and mean blood pressures and also developed hypertrophy [42]. The role of EGF in modulating angiogenesis has shown that pre-treatment of murine bone marrow-derived stromal cells with exogenous EGF can promote neo-vascularization in a mouse model of hind limb ischemia [43]. EGF has also been implicated in the proliferation of SMCs whereby studies have shown that inhibition of EGFR leads to reduction in vascular smooth muscle cell (VSMC) proliferation thus reducing intimal hyperplasia following balloon injury in animal models [44, 45]. Despite a large number of pre-clinical studies on the role of EGF in cardiovascular disease modulation, further clarification on the role of EGF signalling in cardiovascular repair and regeneration are still required in order to develop effective clinical therapies.

1.5 Fibroblast growth factor (FGF)

The fibroblast growth factor (FGF) family can be subdivided into 22 members along with 4 tyrosine kinase receptors [46]. FGFs are considered to be mitogens for multiple cell types whilst also affecting cellular differentiation, migration, and survival [47]. FGFs have been implicated in myocardial regeneration with FGF-1, -2 and -4 reported to promote angiogenesis both *in vitro* and *in vivo* [48, 49], FGF-1 and -2 to confer cardioprotective effects [50], and FGF-2 to promote the activation/differentiation of CSCs [51]. Evidence also shows that cardiac specific overexpression of FGF-1 in animal models provides cardioprotection at the level of the cardiomyocyte by increasing cell viability [52], and FGF-2 has been shown to be a crucial mediator of cardiac hypertrophy via its autocrine/paracrine actions [53].

Harnessing the potential angiogenic capacity of FGFs for cardiovascular treatment has been the main focus of pre-clinical research to date with many studies progressing to advanced-phase clinical trials. Initial clinical trials focused on delivering a single intracoronary infusion of FGF2 to patients with coronary artery disease (CAD). Despite

promising initial phase I results, the FGF Initiating Revascularization Trial (FIRST) phase II trial showed no significant improvement in exercise tolerance or myocardial perfusion but did show a trend toward symptomatic improvement at 90 days follow up [54]. Another promising clinical trial performed perivascular administration of sustained-release FGF-2 capsules concomitantly to coronary artery bypass graft (CABG) and reported that patients had a significant reduction in angina recurrence at long term follow-up at almost 3 years [55]. Subsequent trials have focused on intracoronary delivery of FGF4 in CAD patients, which showed improved regional blood flow [56]. Like VEGF, FGF gene transfer was based on the use of adenoviral vectors as the gene transfer vehicle. However, further clinical studies using the same adenoviral system have failed to reproduce these favourable outcomes, with no significant differences between treatment and placebo identified. However, interestingly when this study was re-classified by gender this revealed that, in women at least, the treatment significantly improved exercise treadmill test time and time to angina [57]. This has subsequently led to the AWARE (Angiogenesis in Women with Angina pectoris who are not candidates for Revascularization) Phase III trial to study the gender-specific response of FGF4 in women with angina. Given that the sustained presence and slow release of growth factors in tissue is important for maintenance of new vasculature, studies which have attempted to harness the potential of FGF have suffered similar issues to those reported for VEGF, with these proteins suffering from short half-lives *in vivo* and viral delivery systems not facilitating adequate expression for a sufficient length of time to achieve beneficial effects [49].

1.6 Transforming Growth factor- β 1 (TGF- β 1)

TGF- β 1 is a cytokine and TGF- β signalling is known to regulate gene networks responsible for fibrosis, angiogenesis, cell proliferation, differentiation, migration and apoptosis [58]. The crucial role of this cytokine in the cardiovascular system was identified when knock-out mice were shown to have severe cardiovascular abnormalities [59]. Many years of investigation have now revealed that TGF- β 1 plays a critical role in the pathogenesis of cardiac disease through modulating inflammatory and reparative responses [60, 61]. Considerable evidence has shown that TGF- β 1 is anti-atherogenic with SMCs obtained from human atherosclerotic plaques shown to be defective in the TGF- β signal pathway [62]. TGF- β has been shown to inhibit proliferation and migration of vSMCs *in vitro* [63], while inhibition of TGF- β signal results in acceleration of atherosclerosis and an unstable plaque phenotype in mouse models [64]. TGF- β has also been shown to maintain the balance between inflammation and fibrosis in atherosclerotic plaques [65].

Despite its cardioprotective role, TGF- β 1 is also a key factor involved in detrimental processes, with prolonged TGF- β signalling negatively affecting post-infarct myocardial remodelling, leading to significant cardiac hypertrophy accompanied by interstitial fibrosis [66]. Promisingly, inhibition of prolonged TGF- β signalling was shown to diminish post-infarct left ventricle dilatation [67] while intracoronary expression of a transgenic antagonist of TGF- β 1 was shown to reduce constrictive remodelling after balloon injury in pigs [68]. However, inhibition of TGF- β signalling at the acute phase of MI has been shown to exacerbate the inflammatory response resulting in more extensive myocardial injury [69]. Therefore while TGF- β signalling may provide an attractive target for modulation of cardiac remodelling, to date this has been hampered by challenges due to the unique biology of this signalling pathway. An important consideration in the development of

future therapies is that the TGF- β signalling must be controlled in tissue-specificity and timing. Indeed we show that CSCs treated with TGF β 1 for 4 days induces their differentiation into cardiomyocyte-like cells, and when TGF β 1 is removed, together with the supplementation of Dickkopf-related protein 1 (Dkk1), CSCs differentiate into beating cardiomyocytes [70] (Figure 1).

2. Exosomes as potential candidates to stimulate endogenous cardiac regeneration

In animal models of acute MI, mesenchymal stem cell (MSC) transplantation significantly reduces infarct size and improves myocardial perfusion [71, 72]. The cardioprotective effect is evident even though the cells were unable to effectively differentiate into cardiomyocytes and less than 6% of the cells were found in the heart 2 weeks after the transplantation [73, 74]. Conditioned medium of Akt-overexpressing MSCs has also shown similar cardioprotective effects and significantly reduced infarct size [75]. Hence the regenerative and cardioprotective effect of MSCs lies in the secretome of the cells and their paracrine abilities [76].

Exosomes are membrane-bounded vesicles that comprise a subset of the extracellular vesicles (EVs) secreted via the fusion of multivesicular bodies (MVBs) with the plasma membrane by almost every cell in the body [77]. They are approximately 70 to 150nm in size, are crucial for cellular communication, and help to orient the cell spatially and temporally [78]. They have been found to carry and secrete proteins, cytokines, growth factors, microRNAs (miRNAs) and other noncoding RNAs, nucleotides and metabolites. The intracellular processes that determine the contents of exosomes are still being studied [79]. Due to their cell-specific cargo and rapid uptake by cells, EVs are being studied as potential candidates for targeted gene and regenerative therapies.

Exosomes have been found to mimic the regenerative effects of MSCs, and therefore are a very attractive candidate as a replacement for stem cell therapies [80]. Exosomes isolated from the conditioned medium of MSCs have been found to have similar regenerative potential and immunomodulatory properties [78]. As with MSC transplantation reducing fibrosis, enhancing angiogenesis, and improving cardiac function after MI, exosomes isolated from MSCs and transplanted intramyocardially have been shown to reduce infarct size and lead to neovascularization, resulting in restoration of contractile function [81]. Moreover, exosomes derived from Akt-modified MSCs and intravenously delivered following AMI in rats, significantly improved cardiac function and promoted new blood vessel formation [82]. Similar effects were also observed from exosomes generated from IPS-derived cardiomyocytes [83] and cardiac progenitors derived from neonatal hearts that were transplanted into rat MI models [84]. Indeed, cardiac progenitor cell-conditioned medium has been shown to encourage tube formation of endothelial cells and to protect cardiomyocytes from stress [85]. Exosomes isolated from cardiac progenitor cells were shown to be enriched with miR210, miR-132, and miR-146a-3p, which had cardioprotective effects, and with miR-451/144 which promoted H9c2 myoblast survival in vitro and cardiomyocyte survival in vivo [86].

The exosome field is in a nascent state. Exosomes could be applied as a combinatorial modality to treat patients in a sustainable manner. The use of exosomes opens up the

possibility of using donor-derived sources in an allogeneic setting without having to surmount immune barriers and age-related functional decline that is seen in an autologous setting. Nonetheless, heterogeneity based on the source and condition of the source material, purification challenges in a good manufacturing practice (GMP) setting for clinical applications, and target specificity in uptake all need to be established before they can be stably and suitably translated to the clinic.

3. Exercise training as a myocardial regenerative therapeutic strategy

The beneficial effects of a program of exercise are well documented and a growing body of evidence has demonstrated the safety and efficacy of vigorous exercise training in the treatment (and prevention) of a number of pathologies [87, 88]. Moreover, a cochrane review found lower all-cause mortality in patients enrolled in a 12-month exercise-based rehabilitation programme [89]. The effect of exercise training as a therapeutic option for the treatment of heart disease has also been assessed [99, 91]. Studies show no harmful effects of exercise training on cardiac function [92], with some reporting improved function [93-95]. During exercise training the normal heart undergoes beneficial physiological ventricular remodelling to adapt to the added workload resulting in increased cardiac mass, function and contractility [96, 97]. On the other hand, pathological cardiac remodelling is detrimental and typically associated with death of cardiomyocytes, fibrotic replacement, cardiac dysfunction, and increased risk of heart failure and sudden death. However while multiple studies have demonstrated a dose-response relationship between exercise and cardiovascular benefit, the optimal dosage, intensity, frequency, and duration of exercise, remain incompletely defined and further randomised controlled trials are needed to support these conclusions.

We have shown that intensity-controlled (relative to VO₂max) treadmill exercise training in adult rats results in improved cardiac function and myocardial mass remodelling through cardiomyocyte hypertrophy, new cardiomyocyte and capillary formation [98]. The new cell formation is due to the activation and ensuing differentiation of the endogenous CSCs [98]. However the beneficial cardiac adaptations produced by 4 weeks of intensity-controlled exercise training were lost after a similar period of de-training [99]. Moreover, endurance swim training in mice induced cardiomyocyte hypertrophy and renewal, which was dependent on a reduction in the expression of the transcription factor C/EBPβ [100]. Furthermore, we have followed a human subject who had previously sustained an MI and undertaken 14-weeks high-intensity interval training, and shown a ~40% reduction of the MI scar measured by cMRI [101]. Together these data support the possibility of myocardial repair and regeneration with exercise training.

The best characterized signalling cascade responsible for mediating physiological cardiac growth is the IGF-1-PI3K(p110α)-Akt pathway. Indeed, increased cardiac IGF-1 expression and activation of the PI3K (p110α) pathway has been implicated in increased cardiomyocyte hypertrophy with endurance exercise in athletes [102]. We identified the up-regulation of neuroregulin, BMP-10, IGF-1, and TGF-β1 in the cardiomyocytes following high intensity treadmill exercise training. Moreover, IGF-1 and neuroregulin increased CSC proliferation, with the activation of their respective receptors and physiologic molecular signalling targets, Akt and STAT-3, respectively [98]. On the other hand, BMP-10 and TGF-

B1 stimulated differentiation of the CSCs into the three main cardiac lineages; cardiomyocytes, vascular smooth muscle and endothelial cells [98]. Other growth factors have also been identified to play a role in exercise-induced cardiac repair, such as elevated levels of angiogenic factor, VEGF-A, after hypoxic exercise training [103]. Furthermore, exercise training up-regulated NRG-1 expression to promote cardiac repair through endogenous regeneration in a rat MI model [104].

Taken together exercise training is a cost-effective intervention for reducing the morbidity and mortality of cardiovascular disease. However, as many heart failure (HF) patients may be unable to exercise at the high intensity dose which leads to effective adaptation and activation of repair mechanisms, understanding the mechanistic underpinnings of exercise may provide biological insights and new therapeutic approaches to promote cardiac regeneration and restore cardiac function.

4. Current clinical evidence that support the use of non-invasive strategies for stimulating endogenous cardiac repair and regeneration

Intensive research has resulted in considerable advances in the discovery of therapeutic targets related to non-invasive strategies for cardiac repair and regeneration. To date a number of growth factors have been evaluated in clinical trials, which have demonstrated safety and potential efficacy for the treatment of heart disease [105]. The increased accessibility and advances in chemical modifications to enhance protein half-life *in vivo* and minimize immunogenicity have also supported the use of growth factors/ cytokines as therapeutic targets for heart repair [106]. However, important challenges still exist in terms of ensuring the reparative mechanisms of these biomolecules and making them clinically viable [107, 108], together this serves to guide the development of future growth factor formulations. Exercise training on the other hand avoids many of the issues involved with delivery of targeted therapeutics and has shown to provide beneficial effects even in the case of chronic heart failure in aged individuals [109]. Exercise training as a regenerative therapeutic strategy may therefore provide a cost-effective, practical strategy for clinical application while the more complex issues of targeted delivery are resolved.

5. Summary

This review highlights the potential of utilising growth factors, cytokines, exosomes and exercise training as non-invasive strategies for stimulating endogenous cardiac repair and regeneration mechanisms. Significant progress in understanding the stimulation, mobilisation and differentiation of CSCs has been made in recent years. Nonetheless, a more comprehensive understanding of CSC biology is required, especially in terms of their long term effectiveness and regenerative potential in the aged-senescent environment. Once this knowledge is in place we will be in a position to obtain maximal potential from these unique cells in a clinical setting.

Conflicts of interest

All authors declare no conflicts of interest.

6. Acknowledgements

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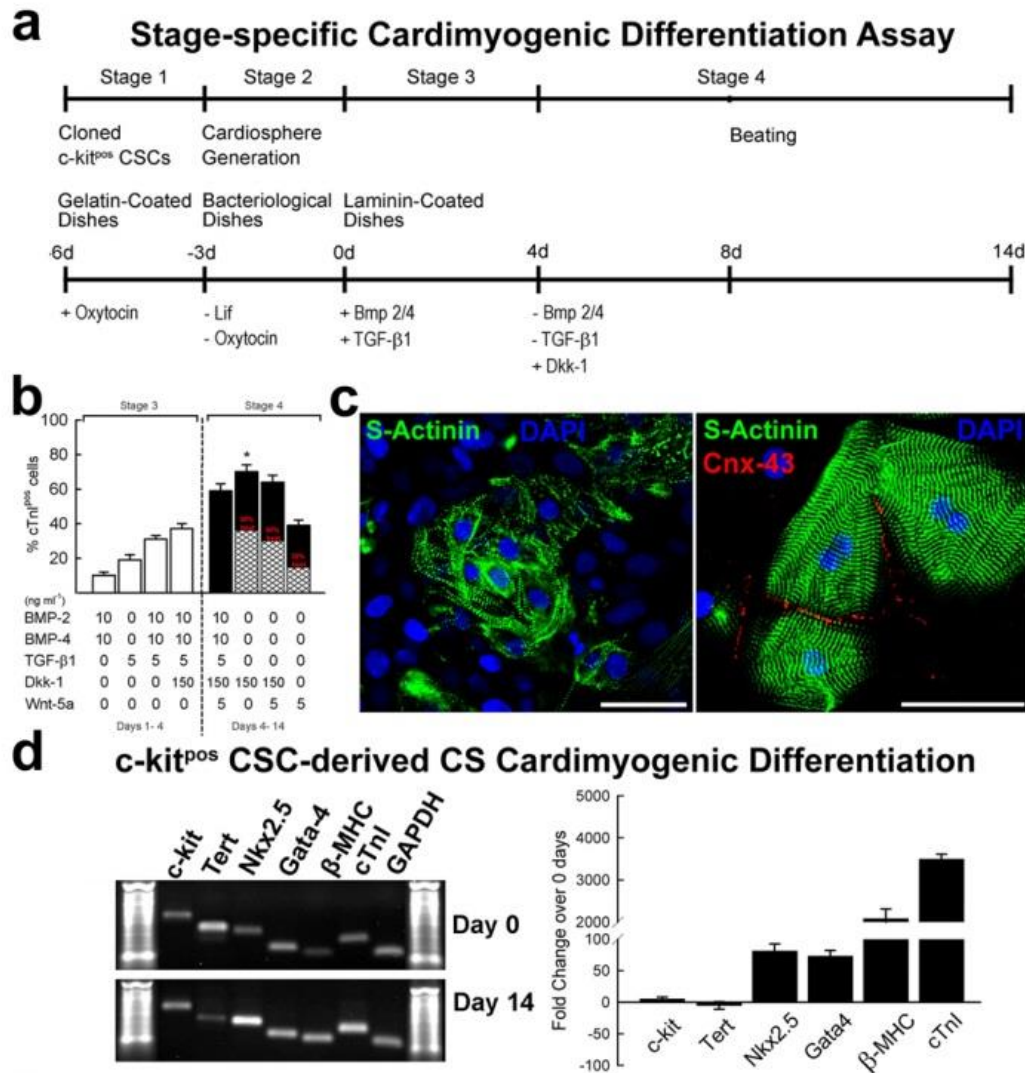


Figure 1. CSCs can differentiate into functionally-competent, striated cardiomyocytes *in vitro* when treated with different growth factors in a stage/sequence-specific manner. (a) Schematic timeline of the stage-specific protocol used for the cardiomycogenic differentiation of c-kit^{pos} CSC cardiospheres. (b) Frequency of cTnI⁺ cells and percentage of beating cells (black bars) after manipulation of the TGF-β/Wnt signaling pathways determined by confocal microscopy and impartial observer counting, as indicated. *P < 0.05 versus all. (c) At days 8-14, differentiated cells stain positive for the cardiomyocyte lineage (S-actinin, green) exhibiting sarcomeric structures (z lines and dots) and gap junction formation (Cnx-43, red) between cells. Scale bars, 50 μm. (d) Real-time qPCR analysis and PCR products after the stage-specific cardiomycocyte differentiation protocol revealed the change in transcripts for the markers c-kit, Tert, Nkx2.5, GATA-4, β-MHC and cTnI in the differentiated cells, relative to 0 day (undifferentiated cells). Data are means ± s.d. of three assays. *Figure adapted from Smith et al. 2014, Nature Protocols.*